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Subject: 48 (Adjuvants)

(54) Vaccine adjuvant.

(57) An adjuvant for potentiating the immunogenicity of an antigen, suitable for manufacture on a commercial scale, is an emulsion having oily particles dispersed in a continuous aqueous phase, which emulsion comprises: an emulsion-forming amount of a non-toxic tetra-polyol or polyoxyethylene-polyoxypropylene (POP-POE) block polymer; optionally, an emulsion-forming amount of a non-toxic metabolizable oil; optionally, an emulsion-stabilizing amount of a glycol ether-based surfactant; and an immunopotentiating amount of a glycopeptide; wherein substantially all of said oily particles have a diameter less than about 800 nm if a POP-POE block polymer is present.

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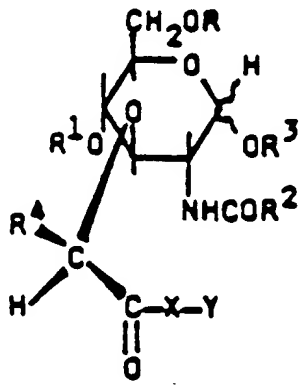
either POP-POE block polymers or tetra-polyols, which maintains the formulations' efficacy, enhances its physical stability, and reduces its sensitivity to refrigeration. Remarkably, such adjuvant emulsions may even be frozen and still retain efficacy.

One aspect of the invention is an adjuvant in the form of an emulsion having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises:
an emulsion-forming amount of a non-toxic tetra-polyol or of a POP-POE block polymer; and
an immunopotentiating amount of a glycopeptide;
wherein substantially all of said oily particles have a diameter less than about 800 nm if a POP-POE block polymer is present.

Another aspect of the invention is an adjuvant in the form of an emulsion having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises an emulsion-forming amount of a non-toxic tetra-polyol; optionally, an emulsion-forming amount of a non-toxic metabolizable oil; optionally, an emulsion-stabilizing amount of a glycol ether-based surfactant; water or aqueous solution, and an immunopotentiating amount of a muramyldipeptide, preferably a derivative of formula I



(I)

and the pharmaceutically acceptable salts thereof, wherein R and R_1 are each independently H or acyl of 1 to 22 carbon atoms, R_2 is alkyl or aryl, optionally substituted with halo, nitro, or lower alkyl, R_3 is H, alkyl, or aryl, R_4 is H or lower alkyl, X is L-alanyl, L-α-aminobutyryl, L-arginyl, L-asparginyl, L-aspartyl, L-cysteinyl, L-glutaminyl, L-glutamyl, glycyl, L-histidyl, L-hydroxyprolyl, L-isoleucyl, L-leucyl, L-lysyl, L-methionyl, L-ornithinyl, L-phenylalanyl, L-prolyl, L-seryl, L-threonyl, L-tyrqsyl, L-tryptophanyl, or L-valyl, and Y is D-glutamine, D-isoglutamine or D-isoasparagine.

Another aspect of the invention is an adjuvant of the type mentioned above, where an emulsion-forming amount of a non-toxic POP-POE block polymer may be substituted for the tetra-polyol, and where substantially all of the oily particles have a diameter less than about 800 nm, preferably less than 300 nm.

Another aspect of the invention is a vaccine, comprising an adjuvant of the invention in combination with an immunogenic amount of an antigen.

Another aspect of the invention is a process for preparing an adjuvant of the invention, which process comprises preparing a first mixture comprising the polymer, oil, surfactant, and water or aqueous solution; emulsifying the mixture to produce an oil-in-water type emulsion having oily particles dispersed in a continuous aqueous phase, wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm; and combining the emulsion with a muramyldipeptide derivative of formula I.

Another aspect of the invention is a kit for extemporaneous preparation of an adjuvant of the invention, which kit comprises: a first container containing an emulsion as described above, and a second container containing the muramyl dipeptide, preferably N-acetylmuramyl-L-threonyl-D-isoglutamine optionally in an aqueous solution or suspension, where the concentrations of the components in each container are selected such that combination of the contents of both containers produces an adjuvant of the invention.

Another aspect of the invention is a kit for the preparation of a vaccine of the invention, which differs from the adjuvant kit described above in that an immunogenic amount of an antigen is added to the second container, or present in a third container.

Another aspect of the invention is a method for inducing an immune response in an animal having an immune system, which method comprises administering a vaccine of the invention.

One aspect of the invention is an adjuvant in the form of an emulsion having oily particles dispersed in

emulsion-forming amount of a non-toxic metabolizable oil; an emulsion-stabilizing amount of a glycol ether-based surfactant; water or aqueous solution; and an immunopotentiating amount of a muramyldipeptide derivative of formula I, wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm. A preferred subgenus is the adjuvant wherein said non-toxic POP-POE block polymer is Pluronic® L121, particularly where said muramyldipeptide derivative of formula I is N-acetylmuramyl-L-threonyl-D-isoglutamine. A preferred class is the adjuvant which includes a non-toxic metabolizable oil, wherein said oil is squalene or squalane. A preferred subclass is the adjuvant wherein said glycol ether-based surfactant is Tween® 80, particularly where said water or aqueous solution comprises isotonic buffered saline.

Another aspect of the invention is an adjuvant in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises a non-toxic POP-POE block polymer in an amount of 0.2 to 49%; a non-toxic metabolizable oil in an amount of 0-15%; a glycol ether-based surfactant in an amount of 0.05-5%; water or aqueous solution; and 0.0001-10% of a muramyldipeptide derivative of formula I, where substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm (% are vol./vol., except for the muramyldipeptide which is wt./vol.). A preferred subgenus is the adjuvant wherein said POP-POE block polymer is Pluronic® L121, particularly where said muramyldipeptide derivative of formula I is N-acetylmuramyl-L-threonyl-D-isoglutamine. A preferred class is the adjuvant which includes a non-toxic metabolizable oil, wherein said oil is squalene or squalane. A preferred subclass is the adjuvant wherein said glycol ether-based surfactant is Tween® 80. A presently preferred embodiment is the adjuvant in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises Pluronic® L121 in an amount of 1-10%; squalane or squalene in an amount of 1-10%; Tween® 80 in an amount of about 0.2%; isotonic buffered saline; and 0.0001-10% N-acetylmuramyl-L-threonyl-D-isoglutamine, wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm.

Another aspect of the invention is a vaccine comprising an adjuvant of the invention in combination with an immunogenic amount of an antigen. Suitably this is a vaccine in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for immunizing an animal, which vaccine comprises an immunogenic amount of an antigen; an emulsion-forming amount of a non-toxic tetra-polyol or a non-toxic POP-POE block polymer; optionally, an emulsion-forming amount of a non-toxic metabolizable oil; optionally an emulsion-stabilizing amount of a glycol ether-based surfactant; water or aqueous solution; and an immunopotentiating amount of a muramyldipeptide, preferably a derivative of formula I. A preferred subgenus is the vaccine which includes a tetra-polyol, especially where said tetra-polyol is Tetronic® 1501. A preferred class is the vaccine wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm. A preferred subclass is the vaccine wherein said muramyldipeptide derivative of formula I is N-acetylmuramyl-L-threonyl-D-isoglutamine. Another preferred subclass is the vaccine wherein said muramyldipeptide derivative of formula I is murabutide. A presently preferred embodiment is the vaccine which comprises: Tetronic® 1501 in an amount of 1-10%; squalane or squalene in an amount of 1-10%; Tween® 80 in an amount of about 0.2%; isotonic buffered saline; and 0.0001-10% N-acetylmuramyl-L-threonyl-D-isoglutamine, especially where substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm. Another preferred subgenus is the vaccine which includes a POP-POE block polymer, wherein said block polymer is Pluronic® L121, wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm. A preferred class is the vaccine wherein said muramyldipeptide derivative of formula I is N-acetylmuramyl-L- threonyl-D-isoglutamine. Another preferred class is the vaccine wherein said muramyldipeptide derivative of formula I is murabutide. A presently preferred embodiment is the vaccine which comprises: Pluronic® L121 in an amount of 1-10%; squalane or squalene in an amount of 1-10%; Tween® 80 in an amount of about 0.2%; isotonic buffered saline; and 0.0001-10% N-acetylmuramyl-L-threonyl-D-isoglutamine.

Another aspect of the invention is a process for preparing the adjuvant or vaccine of the invention, which process comprises mixing together the aqueous phase and the emulsion-forming amount of the non-toxic tetra-polyol or of the POP-POE block polymer so as to form an emulsion.

Another aspect of the invention is a process for preparing an adjuvant of the invention, which process comprises: preparing a first mixture comprising a non-toxic tetra-polyol or a non-toxic POP-POE block polymer, optionally a non-toxic metabolizable oil, optionally a glycol ether- based surfactant, and water or aqueous solution; emulsifying said first mixture to produce an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, wherein substantially all of said oily particles have a diameter less than about 800 nm, preferably less than about 300 nm; and combining said emulsion with a

The term "acyl" refers to radicals of the formula RCO-, where R is H or alkyl as defined above. "Lower acyl" refers to such radicals where R is H or lower alkyl. Examples of acyl include formyl, acetyl, propionyl, butyryl, pentanoyl, hexanoyl, eicosanoyl, and the like. Examples of lower acyl include formyl, acetyl, propionyl, butyryl, pentanoyl, hexanoyl, and the like.

The term "halo" as used herein refers to fluoro, chloro, bromo and iodo.

The term "alkoxy" refers to a radical of the form RO-, where R is lower alkyl or cycloalkyl as defined above.

The term "aryl" refers to aromatic radicals consisting entirely of carbon and hydrogen, containing from 6 to 12 carbon atoms. Examples of aryl groups are phenyl, naphthyl, and the like.

The term "pharmaceutically acceptable salts" refers to acid addition salts of the subject compounds which possess the desired pharmacological activity and which are neither biologically nor otherwise undesirable. These salts are formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid or phosphoric acid; or organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid and the like.

The term "treatment" as used herein covers any treatment of a disease in a bird or mammal, particularly a human, and includes:

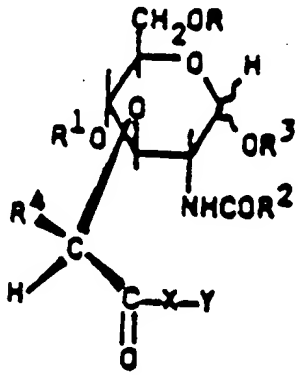
(i) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it;

(ii) inhibiting the disease, i.e., arresting its development; or

(iii) relieving the disease, i.e., causing regression of the disease. (It should be noted that vaccination may effect regression of a disease where the disease persists due to ineffective antigen recognition by the subject's immune system, where the vaccine effectively presents antigen.)

The term "optionally substituted" as applied to aryl radicals in the invention means that the radical may be unsubstituted or substituted with one to three halo, nitro, lower alkyl, or lower alkoxy groups. The optional substituents may be the same or different.

The term "muramyl dipeptide derivative" includes compounds of formula I:



(I)

where R, R_1, R_2, R_3, and R_4 are each independently H, alkyl, acyl, or aryl optionally substituted with halo, nitro, or lower alkyl; X is one or several amino acids, and Y is D-glutamine, D-isoglutamine or D-isoasparagine, which may optionally be esterified or amidated. Preferred compounds are those of Formula 1 wherein R and R_1 are H or acyl of 1 to 22 carbon atoms; R_2 is methyl; R_3 is hydrogen; X is L-alanyl, L-α-aminobutyryl, L-arginyl, L-asparginyl, L-aspartyl, L-cysteinyl, L-glutaminyl, L-glutamyl, glycyl, L-histidyl, L-hydroxyprolyl, L-isoleucyl, L-leucyl, L-lysyl, L-methionyl, L-ornithinyl, L-phenylalanyl, L-prolyl, L-seryl, L-threonyl, L-tyrosyl, L-tryptophanyl, or L-valyl, and Y is D-glutamine or D-isoglutamine. The most preferred MDPs are: N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine; 6-0-stearoyl-N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine; N-acetylmuramyl-L-threonyl-D-isoglutamine; N-acetylmuramyl-L-valyl-D-isoglutamine; N-acetylmuramyl-L-alanyl-D-isoglutamine; N-acetyl-desmethylmuramyl-L-alanyl-D-isoglutamine; N-acetylmuramyl-L-alanyl-D-glutamine butyl ester (murabutide); N-acetylmuramyl-L-seryl-D-isoglutamine; and N-butyrylmuramyl-L-(α-aminobutyryl)-D-isoglutamine. Another useful MDP is N-acetyl-(n-butylmuramyl)-L-α-aminobutyryl-D-isoglutamine.

The term "immunopotentiating amount" refers to the amount of MDP derivative needed to effect an

measuring serum antibody titer, antigen-induced swelling in the skin, and the like.

The term "tetra-polyol" as used herein refers to N,N,N',N'-tetra(polyoxypropylene-polyoxyethylene)-1,2-diaminoethane block polymers. These compounds may be prepared by the process disclosed in U.S. Patent No. 2,979,528, or may be obtained commercially from BASF-Wyandotte under the trademark Tetronic®.

Tetronic® polyols are designated with a three or four digit number which indicates the average molecular weight of the polyoxypropylene (POP) portion and the percentage of the total molecular weight contributed by the polyoxyethylene (POE) portion of the molecule. The first one or two non-zero digits indicate the average molecular weight of the POP section, ranging from 501-1000 for Tetronic® 304 to 6500-7000 for Tetronic® 1501. The last digit indicates the percentage of POE in 10% increments, ranging from 10% for Tetronic® 1501 to 80% for Tetronic® 1508. The characteristics of these compounds are determined by the molecular weight of the POP portion and the amount of POE in the product. Preferred tetra-polyols in the practice of the invention are relatively insoluble in water at 25° C and have low HLB values. The HLB value should preferably be lower than about 5.0. Presently preferred tetra-polyols are Tetronic® 1501, Tetronic® 1301, Tetronic® 1101, and Tetronic® 1502, particularly Tetronic® 1501 and Tetronic® 1301, especially Tetronic® 1501. Other appropriate tetra-polyols with the necessary properties may be prepared using the methods disclosed in U.S. Patent No. 2,979,528, and are to be considered equivalents within the scope of this invention. For example, one could prepare a tetra-polyol with a POP molecular weight of 8,000 and a POE content of 8%. The properties necessary are (i) low HLB value ($\leq$ 5.0, preferably $\leq$ 2.0); (ii) little or no aqueous solubility; (iii) forms stable emulsions with the addition of a glycol ether-based surfactant; and (iv) lack of toxicity.

The term "POP-POE block polymer" refers to a polymer made by the sequential addition of propylene oxide and then ethylene oxide to a low molecular weight, reactive compound, usually propylene glycol. These block polymers can be prepared by the methods set out in U.S. Patent 2,674,619 issued to Lunsted, and are commercially available from BASF-Wyandotte under the trademark Pluronic®. The characteristics of these polyols are determined by the molecular weight of the POP nucleus and of the percentage POE in the product. The POP section imparts hydrophobic characteristics to the block polymer, while the POE section imparts hydrophilic characteristics. Preferred block polymers are determined by the same criteria used to select appropriate tetra-polyols. Preferred block polymers for the practice of the invention are Pluronic® L121 and L101.

Pluronic® polyols are designated by a letter prefix followed by a two or a three digit number. The letter prefixes (L, P, or F) refer to the physical form of each polymer, (liquid, paste, or flakeable solid). The first one or two digits is a code for the average molecular weight of the POP base, while the last digit indicates the amount of POE. For example, Pluronic® L101 is a liquid having a polyoxypropylene base of average molecular weight 3,250, with 10% polyoxyethylene present at the ends of the molecule. The preferred block polymers are those which are liquid over a temperature range between about 15°-40° C. In addition, polymer mixtures of liquid and paste, liquid, paste and flakeable solid or liquid and flakeable solid mixtures which are liquid within the specified temperature range may have utility in this invention.

Preferred block polymers are those having a POP base ranging in molecular weight between about 2250 and 4300 and POE in an amount between about 1 and 30%. More preferred are those polymers wherein POP has a molecular weight falling between 3250 and 4000 and the POE component comprises 10-20%. The Pluronic® polyols L101, L121 and L122 fall within this definition. Most preferred are the polymers wherein POP has a molecular weight of 4000 and POE in an amount of 10% or POP has a molecular weight of 3250 and POE in an amount of 10% eg. Pluronic® polyols L121 and L101 respectively.

An "emulsion-forming amount" of tetra-polyol or POP-POE block polymer is that quantity which will form micelles or an emulsion. For the purposes of the invention this is an amount between 0.2% and 49% by volume. A more preferred amount is from 0.25% to 20%, and about 1-5% is even more preferred. A concentration of 1-2.5% is presently most preferred.

The term "surfactant" refers to non-toxic surface active agents capable of stabilizing the emulsion. There are a substantial number of emulsifying and suspending agents generally used in the pharmaceutical sciences. These include naturally derived materials such as gums, vegetable protein, alginates, cellulose derivates, phospholipids (whether natural or synthetic), and the like. Certain polymers having a hydrophilic substituent on the polymer backbone have surfactant activity, for example, povidone, polyvinyl alcohol, and glycol ether-based compounds. Compounds derived from long chain fatty acids are a third substantial group of emulsifying and suspending agents usable in this invention. Though any of the foregoing surfactants can be used so long as they are non-toxic, glycol ether-based surfactants are preferred. Preferred surfactants are non-ionic. These include polyethylene glycols (especially PEG 200, 300, 400, 600 and 900), Span®, Arlacel®, Tween®, Myrj®, Brij® (all available from ICI, America's Inc., Wilmington,

TC-100 computing autocorrelator.

Biological activity may be assayed using standard laboratory techniques, e.g., by vaccinating a standard laboratory animal (e.g., a Guinea pig) with a standard antigen (e.g., BSA or DNP-BSA) using a test adjuvant formulation. After allowance of time for boosting the vaccination, and time for immunization to occur, the animal is challenged with the standard antigen and the results measured. The response may be quantified by any measure accepted in the art for measuring immune responses, e.g., in terms of serum antibody titer against the standard antigen (for humoral immunity) and skin test reaction (for cell-mediated immunity).

ADMINISTRATION

It will be apparent to one of ordinary skill in the art that the precise amounts of MDP derivative and antigen needed to produce a given effect will vary with the particular compounds and antigens, and with the size, age, and condition of the subject to be treated. Thus, it is impossible to state exactly the amounts needed: however, these amounts can easily be determined using methods known to those of ordinary skill in the art.

The adjuvants and vaccines of the invention are generally administered by injection, particularly intramuscular injection, preferably into a large muscle.

In general, an initial vaccination is administered using the desired antigen and the formulation of the invention. The vaccination is "boosted" several weeks later (usually 2-6 weeks, for example, 4-6 weeks) using a vaccine of the invention with or without (preferably with) the MDP component. Generally, 1-2 mL of a vaccine (such as are described in the Examples below) is administered to a human subject in the pratice of the invention.

The following examples are presented as an aid to those of ordinary skill in the art, and are not to be considered as a limitation of the invention in any way.

EXAMPLE 1

(Immunogenicity)

(A) Preparation of Formulations:

Adjuvant formulations were prepared as follows for assay of biological activity. Each emulsion was prepared at 2X concentration prior to combination with a 2X solution of antigen.

Formulation 1 (from Allison, U.S. 4,606,918): 5.0% Pluronic® L121, 10% squalane, 0.4% Tween® 80, qs phosphate buffered saline (pH 7.4); the components were added to a test tube and vortex-mixed until a milky emulsion was obtained. This formulation was prepared immediately prior to administration.

Formulation 2 (Formulation 1 with refrigeration): 5.0% Pluronic® L121, 10% squalane, 0,4% Tween® 80, qs phosphate buffered saline (pH 7.4); the components were added to a test tube and vortex-mixed until a milky emulsion was obtained. The formulation was then refrigerated at 4° C beginning one day prior to administration.

Formulation 3 (tetra-polyol formulation of the invention): 5.0% Tetronic® 1501, 10% squalane, 0.4% Tween® 80, qs phosphate buffered saline (pH 7.4); the components were added to a test tube and vortex-mixed until a milky emulsion was obtained.

Formulation 4 (microfluidized POP-POE adjuvant of the invention): 5.0% Pluronic® L121, 10% squalane, 0.4% Tween® 80, qs phosphate buffered saline (pH 7.4); the components were added to a test tube and vortex-mixed until a milky emulsion was obtained. This emulsion was then passed through a Microfluidizer® four times. This formulation was refrigerated with Formulation 2.

To each formulation was then added solid N-acetylmuramyl-L-threonyl-D-isoglutamine (Thr-MDP) to a concentration of 500 µg/mL, to form the complete adjuvant "concentrate." The concentrate was then mixed with a 2X concentration solution of antigen (ovalbumin in saline, 1 mg/mL) to form a test vaccine.

(B) Particle Size:

Particle size distributions were analyzed by optical microscopy (Leitz Ortholux II POL-BK polarized light microscope), transmission electron microscopy (TEM, using a Hitachi model HS-8-1), and laser photon correlation spectroscopy (PCS, using a Nicomp Model 200 laser particle sizer, with a model TC-100 computing autocorrelator). Particle size distributions were determined for top layers and bottom layers separately. Samples analyzed by TEM and PCS were diluted 1:100 or greater before analysis. The results demonstrated that Formulations 1-3 exhibited particle sizes ranging from < 0.1 µm to about 25 µm. Formulatio 4 exhibited particle sizes ranging from < 0.1 µm to about 0.3 µm (300 nm).

EXAMPLE 3

(Formulations)

Exemplary adjuvant formulations were prepared as follows:

(A) Tetronic®/Thr-MDP:	
Tetronic® 1501	2.5 g
Squalane	5.0 g
Tween® 80	0.2 g
Thr-MDP	250.0 mg
Phosphate buffered saline	qs to 100.0 mL

The Tetronic® 1501, squalane, and Tween® 80 are placed in an appropriate vessel with 85 mL of phosphate buffered saline (PBS) and are mixed with a mechanical mixer (Greerco Homogenizer-Mixer, model #1L-79, Greerco Corp., Hudson, New Hampshire) at about 4750 rpm for about 30-60 minutes. Then, the Thr-MDP (N-acetylmuramyl-L- threonyl-D-isoglutamine) and remaining 15 mL of PBS are stirred in, producing an adjuvant formulation of the invention.

(B) Similarly, proceeding as in part (A) above but substituting N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (Abu-MDP), 6-O-stearoyl-N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (Abu-MDP stearate), N-acetylmuramyl-L-valyl-D-isoglutamine (Val-MDP), N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), N-acetyldesmethylmuramyl-L-alanyl-D-isoglutamine (desMe-MDP), N-acetylmuramyl-L-alanyl-D-glutamine butyl ester (murabutide), n-butyrylmuramyl-L-(α-aminobutyryl)-D-isoglutamine, and N-acetylmuramyl-L-seryl-D-isoglutamine (Ser-MDP), for the Thr-MDP, the corresponding adjuvant formulations are prepared.

(C) Pluronic®/Thr-MDP:	
Pluronic® L121	2.5 g
Squalane	5.0 g
Tween® 80	0.2 g
Thr-MDP	250.0 mg
Phosphate buffered saline	qs to 100.0 mL

The Pluronic® L121, squalane, and Tween® 80 are placed in an appropriate vessel with 85 mL of phosphate buffered saline (PBS) and are mixed with a mechanical mixer (e.g., Greerco Homogenizer-Mixer) at about 4750 rpm for about 5-10 minutes. Then, the Thr-MDP (N-acetylmuramyl-L-threonyl-D-isoglutamine) and remaining 15 mL of PBS are stirred in. The resulting emulsion is then processed through a Microfluidizer® (Microfluidics Corp.) for at least 4 cycles to provide an adjuvant formulation of the invention. Alternatively, the MDP is added after the microfluidization step.

(D) Similarly, proceeding as in part (C) above but substituting N-acetylmuramyl-L-α-aminobutyryl-D-iso glutamine (Abu-MDP), 6-O-stearoyl-N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (Abu-MDP stearate),

L121 and 0.4% polysorbate 80 in phosphate buffered saline, were used in the test, having been prepared as for Formulation 4 (Example 1). One emulsion was stored frozen for seven days before use, while the other was freshly prepared and kept at room temperature. On the day vaccines were prepared, Thr-MDP was added to the fresh and thawed emulsions. Equal volumes of 2X concentrated ovalbumin were added to the 2 lots of emulsions just before the vaccines were used to immunize groups of 8 female guinea pigs. The final concentrations of the constituents of the vaccines were: Phosphate buffered saline 92.33%; Squalane 5%; Pluronic® L121, 2.5%; Polysorbate 80, 0.17%; Thr-MDP 250 µg/ml; and Ovalbumin 1.0 mg/ml.

Guinea pigs were vaccinated on days 0 and 28 with 0.2 ml of vaccine per animal, bled on days 28 and 42, and skin tested with 10 µg of ovalbumin on day 42.

The results obtained, as shown below, show that the efficacy of the frozen material was equivalent to that of the freshly prepared emulsion.

TABLE 1

ANTIBODY TITRES[a]					
			28 Days	42 Days	
Group	No. of Animals	Vehicle Preparation	Mean Titre ± SE	Mean Titre ± SE	Equivalent Dilution[b]
1	8	Fresh	4.6 ± 0.2	9.0 ± 0.1	18,837
2	8	Frozen	5.1 ± 0.3	8.9 ± 0.1	17,830

[a] Titres are expressed as $\log_3$ of the reciprocal of the serum dilution giving an optical density reading of 0.5 absorbance units, under the conditions of the assay.
[b] Titre expressed as the reciprocal of the mean serum dilution.

TABLE 2

DELAYED HYPERSENSITIVITY SKIN REACTIONS				
Group	No. of Animals	Vehicle Preparation	Mean Diameter (mm ± SE)	
			24 Hr	48 Hr
1	8	Fresh	14.3 ± 0.7	11.0 ± 1.7
2	8	Frozen	13.3 ± 2.3[a]	11.4 ± 2.9[a]

[a] Includes one animal which had no response and may not have been skin tested.

EXAMPLE 5

Hepatitis Virus Vaccine

Groups of 8 female Hartley guinea pigs were immunized subcutaneously with a vaccine consisting of 0.5 µg or 0.1 µg of Hepatitis B virus surface antigen (HBsAg) in microfluidized adjuvant (prepared as for Formulation 4, Example 1, without refrigeration) or adsorbed to alum (commercially available hepatitis vaccine). The HBsAg in saline and HBsAG adsorbed to alum were provided by Merck Sharpe and Dohmé Research Laboratories. The adjuvant preparation consisted of 92.33% PBS, 5% squalane, 2.5% Pluronic L121, 0.17% polysorbate 80, 100 µg/ml Thr-MDP, and either 1.0 µg/ml or 0.2 µg/ml of HBsAg. Each animal

TABLE

MEAN ANTI-HA TITRES OF MICE 13 WEEKS FOLLOWING IMMUNIZATION						
Group	HA Dose (µg)	Boost at 3 wks	Vehicle	Mean Titre ± SE		
				A/Taiwan	A/Leningrad	B/Ann Arbor
1	0	+	Adjuvant	<3.0 ± 0[b,c]	<3.0 ± 0[b]	<3.0 ± 0[b]
2	0.01	+	Adjuvant	9.1 ± 0.1	8.0 ± 0.2	6.8 ± 0.2
3	0.01	-	Adjuvant	8.8 ± 0.2	7.8 ± 0.3	5.9 ± 0.3

[a] Titre is $\log_3$ of the reciprocal of the serum dilution giving an optical density of 0.5 absorbance units.
[b] Lowest dilution tested was 1/27, i.e., $1/3^3$
[c] Sera of 2 animals had titres of 3.1 and 3.2, while for the remaining B sera no antibody was detectable.

EXAMPLE 7

The ovalbumin vaccine of Example 4 was prepared as described in Formulation 4, Example 1, but without the Tween 80.

EXAMPLE 8

The ovalbumin vaccine of Example 4 was prepared as described in Formulation 4, Example 1, but the vaccine composition contained only 1.25% Pluronics® L121.

EXAMPLE 9

Other Vaccines

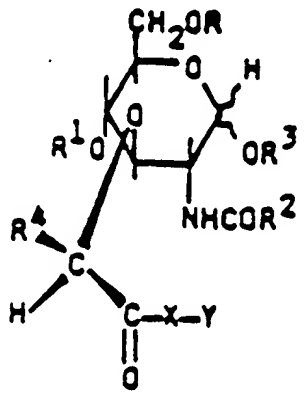
The ovalbumin vaccine of Example 4 was prepared as described in Formulation 4, Example 1, but using the following antigens in place of ovalbumin:
HIV (Human immunodeficiency virus)
Plasmodium yoelii peptides
Influenza viruses (A and B types)
Adenoviruses
Herpes simplex virus type 1, glycoprotein gD1
Melanoma antigens (mouse and human)
Foot and mouth disease virus
Hepatitis B virus surface antigens
Hepatitis A virus
Para-influenza 3 glycoproteins
SIV (simian immunodeficiency virus)
Shistoma mansoni cercaria
Folate hydrolase
Polio virus
Mouse idiotype antibody

In the tests reported above, no significant side effects were observed.

Claims

1. An adjuvant in the form of an emulsion having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises:
an emulsion-forming amount of a non-toxic tetra-polyol or of a POP-POE block polymer; and
an immunopotentiating amount of a glycopeptide;
wherein substantially all of said oily particles have a diameter less than about 800 nm if said POP-POE block polymer is present.

2. An adjuvant according to Claim 1 in the form of an emulsion having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises:
an emulsion-forming amount of a non-toxic tetra-polyol;
optionally, an emulsion-forming amount of a non-toxic metabolizable oil;
an emulsion-stabilizing amount of a glycol ether-based surfactant;
water or aqueous solution; and
an immunopotentiating amount of a muramyldipeptide derivative of formula I



(I)

and the pharmaceutically acceptable salts thereof, wherein
R and R_1 are each independently H or acyl of 1 to 22 carbon atoms;
R_2 is alkyl or aryl, optionally substituted with halo, nitro, or lower alkyl;
R_3 is H, alkyl, or aryl;
R_4 is H or lower alkyl;
X is L-alanyl, L-α-aminobutyryl, L-arginyl, L-asparginyl, L-aspartyl, L-cysteinyl, L-glutaminyl, L-glutamyl, glycyl, L-histidyl, L-hydroxyprolyl, L-isoleucyl, L-leucyl, L-lysyl, L-methionyl, L-ornithinyl, L-phenylalanyl, L-prolyl, L-seryl, L-threonyl, L-tyrosyl, L-tryptophanyl, or L-valyl; and
Y is D-glutamine, D-isoglutamine or D-isoasparagine.

3. The adjuvant of Claim 1 or 2 wherein said tetra-polyol is Tetronic® 1501.

4. The adjuvant of Claim 3 which includes a non-toxic metabolizable oil, wherein said oil is squalene or squalane.

5. The adjuvant of Claim 3 or 4 wherein said glycol ether-based surfactant is Tween® 80.

6. The adjuvant of Claim 3, 4 or 5 wherein said water or aqueous solution comprises isotonic buffered saline.

7. The adjuvant of any one of Claims 3-6 wherein substantially all of said oily particles have a diameter less than about 800, preferably less than about 300 nm.

8. The adjuvant of any one of Claims 1-7 wherein said muramyl- dipeptide derivative of formula I is:
N-acetylmuramyl-L-threonyl-D-isoglutamine,
N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine,
6-O-stearoyl-N-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine,
N-acetylmuramyl-L-valyl-D-isoglutamine,
N-acetylmuramyl-L-alanyl-D-isoglutamine,
N-acetyl-desmethylmuramyl-L-alanyl-D-isoglutamine,

16. The adjuvant of any one of Claims 11-15 wherein said water or aqueous solution comprises isotonic buffered saline.

17. An adjuvant according to Claim 11 in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises:
a non-toxic POP-POE block polymer in an amount of 0.2 to 49%;
a non-toxic metabolizable oil in an amount of 0-15%;
a glycol ether-based surfactant in an amount of 0.05-5%;
water or aqueous solution; and
0.0001-10% a muramyldipeptide derivative of formula I
wherein substantially all of said oily particles have a diameter less than about 800 nm.

18. An adjuvant according to Claim 17 in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for potentiating the immunogenicity of an antigen, which adjuvant comprises:
Pluronic® L121 in an amount of 1-10%;
squalane or squalene in an amount of 1-10%;
Tween® 80 in an amount of about 0.2%;
isotonic buffered saline; and
0.0001-10% N-acetylmuramyl-L-threonyl-D-isoglutamine, wherein substantially all of said oily particles have a diameter less than about 800 nm.

19. A vaccine in the form of an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, for immunizing an animal, which vaccine comprises:
an immunogenic amount of an antigen; and
an adjuvant according to any one of the Claims 1-18.

20. A process for preparing the adjuvant or vaccine according to any one of the preceding claims, which process comprises mixing together the aqueous phase and the emulsion-forming amount of the non-toxic tetra-polyol or of the POP-POE block polymer so as to form an emulsion.

21. A process according to Claim 20 wherein the glycopeptide is added to the mixture after the emulsion is formed.

22. A process according to Claim 20 or 21 wherein a vaccine is formed by adding an antigen to the emulsion.

23. A process according to any one of the Claims 20-22 wherein the emulsification yields oily particles substantially all of which have a diameter less than about 800 mm.

24. A process according to Claim 23, wherein a Microfluidizer® is used as a mixer.

25. A kit for extemporaneous preparation of an adjuvant according to any one of Claims 1-18, which kit comprises:
a first container containing the emulsion of the tetra-polyol or POP-POE polymer in the aqueous phase; and
a second container containing the glycopeptide.

26. A kit according to Claim 25 for extemporaneous preparation of an adjuvant of the invention, which kit comprises:
a first container containing an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, where said emulsion comprises Tetronic® 1501 or Pluronic® L121, squalane or squalene, Tween® 80, and isotonic buffered saline, where substantially all of said oily particles have a diameter less than about 800 nm; and
a second container containing N-acetylmuramyl-L-threonyl-D-isoglutamine;
where the concentrations of the components in each container are selected such that combination of the contents of both containers produces a formulation comprising Tetronic® 1501 or Pluronic® L121 in an amount of 1-30%, squalane or squalene in an amount of 1-30%, Tween® 80 in an amount of about 0.2-5%, 0.0001-30% N-acetylmuramyl-L-threonyl-D-isoglutamine, and isotonic buffered saline.

27. A kit for extemporaneous preparation of a vaccine according to Claim 19, which kit comprises:
a first container containing the emulsion of the tetra-polyol or POP-POE block polymer in the aqueous phase; and
a second container containing the antigen;
wherein the glycopeptide may be present in a third container, or in the first or second containers.

28. A kit according to Claim 27 for extemporaneous preparation of a vaccine of the invention, which kit comprises:
a first container containing an oil-in-water type emulsion, having oily particles dispersed in a continuous aqueous phase, where said emulsion comprises Tetronic® 1501 or Pluronic® L121, squalane or squalene,